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Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase)

D.Michael Payne[2,4], Anthony J.Rossomando[2], Paul Martino[3], Alan K.Erickson[1], Jeng-Horng Her[2], Jeffrey Shabanowitz[3], Donald F.Hunt[3], Michael J.Weber[2] and Thomas W.Sturgill[1,5]

[1]Departments of Internal Medicine and Pharmacology, [2]Department of Microbiology and Cancer Center and [3]Department of Chemistry, University of Virginia, Charlottesville, VA 22908, USA

[4]Present address: Department of Animal Science, Auburn University, Auburn, AL 36849, USA

[5]Corresponding author

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Mitogen-activated protein kinase (MAP kinase) is a 42 kd serine/threonine protein kinase whose enzymatic activity requires phosphorylation of both tyrosyl and threonyl residues. As a step in elucidating the mechanism(s) for activation of this enzyme, we have determined the sites of regulatory phosphorylation. Following proteolytic digestion of ^{32}P-labeled pp42/MAP kinase with trypsin, only a single phosphopeptide was detected by two-dimensional peptide mapping, and this peptide contained both phosphotyrosine and phosphothreonine. The amino acid sequence of the peptide, including the phosphorylation sites, was determined using a combination of Fourier transform mass spectrometry and collision-activated dissociation tandem mass spectrometry with electrospray ionization. The sequence for the pp42/MAP kinase tryptic phosphopeptide is similar (but not identical) to a sequence present in the ERK1- and KSS1-encoded kinases. The two phosphorylation sites are separated by only a single residue. The regulation of activity by dual phosphorylations at closely spaced threonyl and tyrosyl residues has a functional correlate in p34^{cdc2}, and may be characteristic of a family of protein kinases regulating cell cycle transitions.

Key words: electrospray ionization/mass spectrometry/mitogenesis/phosphotyrosine/protein kinase

Introduction

Serine/threonine-specific protein kinases have been identified as regulatory effectors in numerous cellular activities, but the cell-surface receptors which initiate changes in these activities are often tyrosine protein kinases. Thus, understanding the regulatory cascades initiated at the cell surface requires identification of serine/threonine protein kinases whose enzymatic activation is regulated by tyrosine phosphorylation. We refer to these enzymes as 'switch' kinases because they switch the signalling language from tyrosine phosphorylation to serine/threonine phosphorylation. Mitogen-activated protein kinase (MAP kinase) was the first such 'switch' kinase to be identified (Ray and Sturgill, 1987, 1988b).

MAP kinase is a 42 kd serine/threonine-specific protein kinase which rapidly becomes phosphorylated on both tyrosine and threonine and becomes concurrently activated after quiescent cells are treated with stimulatory agents. Such agents include polypeptide growth factors whose receptors are protein tyrosine kinases, such as insulin (Ray and Sturgill, 1987), epidermal growth factor (Rossomando *et al.*, 1989), and platelet-derived growth factor (L'Allemain *et al.*, 1991) as well as agonists for the serine/threonine-specific protein kinase C (Erickson *et al.*, 1990). Treatment of secretory tissues with secretagogues also can activate MAP kinase (Ely *et al.*, 1990). MAP kinase has been found to be identical (or very closely related) to one of the phosphoforms of pp42, a protein which becomes tyrosine phosphorylated in response to various mitogenic agents and oncogenes (Rossomando *et al.*, 1989, and references therein).

In vitro, MAP kinase is able to phosphorylate microtubule-associated protein-2 (Ray and Sturgill, 1988a), *Xenopus* ribosomal S6 kinase II (Sturgill *et al.*, 1988), and myelin basic protein (MBP) (Erickson *et al.*, 1990), whereas the more commonly used kinase substrates, such as histone and casein, are not appreciably phosphorylated by this kinase. Recently, the specific site in MBP phosphorylated by MAP kinase was identified as Thr97. Although cAMP-dependent protein kinase, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase can all utilize MBP as an *in vitro* substrate, none modifies this residue (Erickson *et al.*, 1990). Thus, phosphorylation of a novel site in MBP further serves to distinguish MAP kinase from members of these other kinase families.

The *in vivo* functions of MAP kinase remain to be firmly established, but a variety of evidence suggests that MAP kinase functions in a kinase cascade resulting in activation of an S6 kinase and phosphorylation of ribosomal protein S6 (Sturgill *et al.*, 1988; Gregory *et al.*, 1989; Ahn and Krebs, 1990a,b; L'Allemain *et al.*, 1991). This evidence includes the above-mentioned ability of MAP kinase to phosphorylate and reactivate S6 kinase which had previously been deactivated by phosphatase treatment (Sturgill *et al.*, 1988; Gregory *et al.*, 1989; see also Ahn and Krebs, 1990a,b), as well as our recent finding that a variant cell line defective in TPA-induced S6 kinase activity (Erikson *et al.*, 1988) is similarly defective in TPA-induced MAP kinase activity (L'Allemain *et al.*, 1991).

Since MAP kinase can be activated by agents which stimulate protein tyrosine phosphorylation as well as by the phorbol esters, which activate protein kinase C (a serine/threonine protein kinase), the suggestion has been made that MAP kinase functions at a point of convergence between different signaling pathways (Rossomando *et al.*, 1989). This suggestion has been supported by the finding that MAP kinase requires phosphorylation on both tyrosyl

and threonyl residues for enzymatic activity: MAP kinase can be completely inactivated by treatment with either CD45 or phosphatase 2A, accompanied by essentially complete removal of phosphate from either tyrosyl or threonyl residues, respectively (Anderson *et al.*, 1990).

By contrast, virtually nothing is known about the kinases which phosphorylate and activate MAP kinase, nor about the mechanisms of their regulation. Elucidation of the protein sequence surrounding each of the regulatory sites of phosphorylation would aid in the identification of these kinases, and would provide additional insight into the structural requirements for MAP kinase activation. We now report results of studies in which the phosphorylation sites in murine MAP kinase were determined by peptide mapping and mass spectrometry. We find that both the tyrosine and threonine phosphorylations reside in a single tryptic phosphopeptide, the amino acid sequence of which was determined by tandem mass spectrometry. The sequence obtained for this peptide is similar (but not identical) to a corresponding sequence predicted for an insulin-stimulated microtubule-associated protein-2 kinase encoded by a rat cDNA (ERK1) (Boulton *et al.*, 1990a). The tyrosine and threonine phosphorylation sites are separated by only a single residue. Thus MAP kinase regulation resembles that of mammalian $p34^{cdc2}$ in that both are regulated by closely spaced dual phosphorylations at tyrosine and threonine.

Results

Analytical peptide mapping of [^{32}P]MAP kinase by two-dimensional HVE/TLC

MAP kinase isolated from ^{32}P-labeled, insulin-stimulated Swiss 3T3 cells contains phosphothreonine and phosphotyrosine (Ray and Sturgill, 1988b). Consistent with those previous results, purified [^{32}P]MAP kinase (Figure 1A, arrow) from PDB-stimulated EL4 cells also contains both phosphotyrosine and phosphothreonine, and no detectable phosphoserine (Figure 1B). When [^{32}P]MAP kinase was digested with trypsin and the products resolved by HVE/TLC, only one ^{32}P-labeled phosphopeptide was detected (Figure 1C). This phosphopeptide also contained both phosphotyrosine and phosphothreonine (Figure 1D).

Preparation of MAP kinase for sequence analysis

pp42/MAP kinase is nonabundant and inactive in quiescent cells, requiring mitogenic stimulation of cells prior to purification. MAP kinase is also readily inactivated by phosphatases, and as a trace protein, is prone to severe handling losses. Thus, preparation of amounts of the phosphopeptide sufficient for sequencing required the use of mass cell culture, some modifications to our previously described purification procedure (Ray and Sturgill, 1988a), and development of a phosphopeptide isolation and sequencing strategy of general utility (Erickson *et al.*, 1990), as outlined below.

MAP kinase was purified for sequencing from the 25 000 *g* supernatant of lysates from stimulated EL4.IL-2 cells using the following sequential steps (see Materials and methods): (i) batch adsorption to phenyl-Sepharose, (ii) gradient elution from MonoQ, (iii) gradient elution from phenyl-Superose and (iv) SDS−PAGE. Because of the presence of other MBP (and microtubule-associated protein-2) phosphotransferases in the cytosol and the scarcity of the MAP kinase protein, we have not attempted to calculate specific activity at each stage of our purification. Rather, we have utilized [^{32}P]MAP kinase, purified from the same source (see Materials and methods), in a utilitarian fashion to monitor recovery and optimize the various steps. Use of [^{32}P]MAP kinase as a tracer to monitor binding and recovery during preparative phenyl-Superose chromatography is illustrated in Figure 2. Essentially all of the [^{32}P]MAP kinase bound to the column and co-eluted with MAP kinase activity in fractions 23−27; elution at that position in the gradient was as expected, based on the calculated scale-up from analytical to preparative columns (Erickson *et al.*, 1990). Based on the recovery of tracer

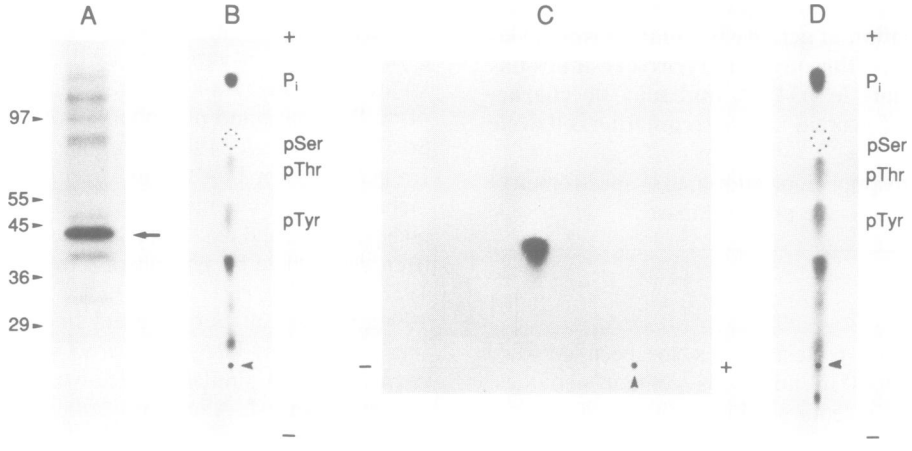

Fig. 1. Phosphopeptide mapping and phosphoamino acid analysis of pp42/MAP kinase. (**A**) SDS−PAGE analysis of ^{32}P-labeled MAP kinase from EL4 cells following phorbol ester stimulation and purification by sequential DE-52 and phenyl-Superose chromatography (see Materials and methods). Following autoradiography (4°C), the MAP kinase band (arrow) was excised from the wet gel and eluted for subsequent analyses (B−D). (**B**) A portion of the gel-purified [^{32}P]MAP kinase was subjected to phosphoamino acid analysis. (**C**) The remainder of the purified [^{32}P]MAP kinase was digested with trypsin and analyzed by two-dimensional phosphopeptide mapping as described (Erickson *et al.*, 1990). (**D**) The purified tryptic phosphopeptide was recovered from the thin-layer plate and subjected to phosphoamino acid analysis. Autoradiographic detection for B−D was performed at −70°C with intensifying screens; the positions of the phosphorylated amino acid standards in (B) and (D) were visualized by ninhydrin staining.

[^{32}P]MAP kinase, overall recovery of MAP kinase, up to and including the preparative phenyl-Superose step, was 65%.

Attempts at further chromatographic steps resulted in a significant decrease in recovery, as in our experience, pp42/MAP kinase at this stage of purity was subject to severe handling losses in the absence of either carrier or detergent, regardless of the materials it contacted (polypropylene, Teflon, etc.). Thus, we employed preparative SDS−PAGE as the final purification step. Five large-scale preparations of phenyl-Superose purified MAP kinase (representing enzyme from 1.3×10^{10} stimulated EL4 cells) were pooled, along with [^{32}P]MAP kinase as tracer, and further fractionated by preparative SDS−PAGE; an autoradiogram of the wet gel is shown as an inset in Figure 2. The MAP kinase band, representing the major phosphoprotein present, was carefully excised, and the protein was eluted and precipitated as described (see Materials and methods).

Peptide purification by sequential HVE/TLC and reverse phase HPLC

After treatment of the purified protein with trypsin, the products of the digestion were resolved by preparative HVE/TLC, using conditions equivalent to those employed to generate Figure 1C. The phosphopeptide was recovered from the plates (see Materials and methods) and further purified by reverse phase chromatography. The absorbance of the effluent at 214 nm and radioactivity in corresponding fractions are shown in Figure 3. The tracer ^{32}P-labeled phosphopeptide eluted with a clearly resolved absorbance peak. The amount of peptide recovered in this peak was estimated to be 30−50 pmol. This estimate is based on comparisons of the peak height with those obtained from known amounts of standard peptides having similar size and composition (data not shown); these peptides were selected once the sequence of the MAP kinase peptide was determined. A control was performed using tracer [^{32}P]MAP kinase alone but with conditions otherwise identical to those described above. With only tracer present, no UV-absorbing peak co-eluted with the [^{32}P]phosphopeptide and no peptides were detectable by mass spectrometry (data not shown). This control demonstrated that the separation procedures resolved the phosphopeptide from carrier and trypsin degradation products. It is to be

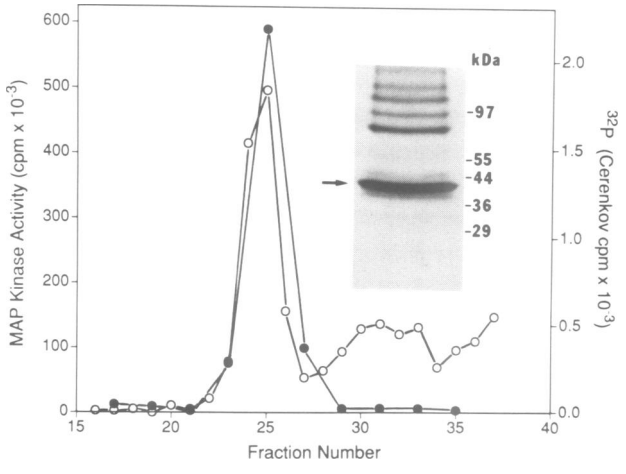

Fig. 2. Preparative phenyl-Superose chromatography of pp42/MAP kinase. pp42/MAP kinase from 10^9 PDB-stimulated EL4 cells was partially purified by phenyl-Sepharose and Mono-Q chromatography, then pooled with tracer [^{32}P]MAP kinase (to determine recovery) and applied to a preparative phenyl-Superose column. Fractions were analyzed for radioactivity (○) and MAP kinase activity (●); the fractions comprising the elution gradient are shown. Recovery of MAP kinase was estimated to be 80% for this chromatographic step. Inset: five preparations of phenyl-Superose purified pp42/MAP kinase (representing a total of 1.3×10^{10} cells) were pooled, along with tracer [^{32}P]MAP kinase and further purified by SDS−PAGE. An autoradiograph of the wet gel is shown; the MAP kinase band (arrow) was excised and the eluted protein was used to generate phosphopeptide for sequence analysis.

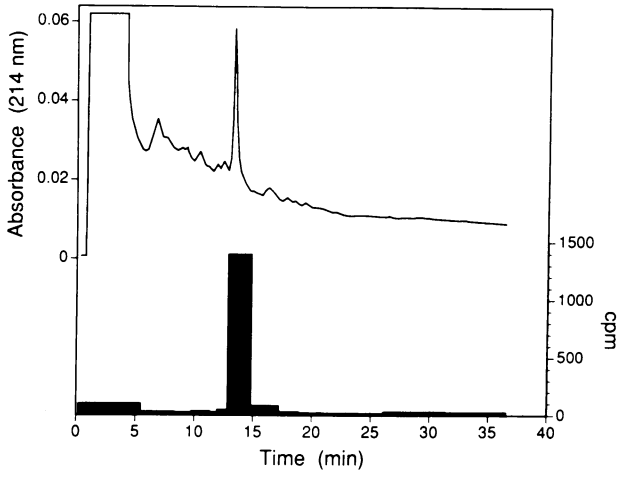

Fig. 3. Reverse phase HPLC purification of the HVE/TLC-purified tryptic phosphopeptide from pp42/MAP kinase. Purified MAP kinase (Figure 2, inset) was eluted from the gel, digested with trypsin and the phosphopeptide purified by HVE/TLC (see Figure 1C and Materials and methods). Material recovered from the thin-layer plates was further purified by HPLC using a C_4 reverse phase column. Recovery of the phosphopeptide was 98% (based on radioactivity).

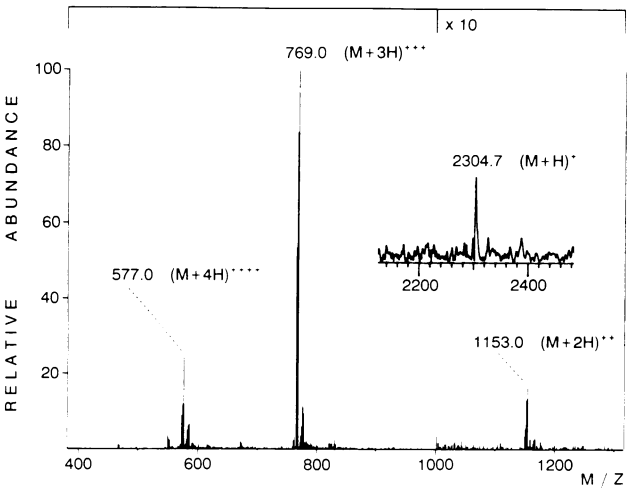

Fig. 4. Particle bombardment and electrospray ionization mass spectra recorded on the purified tryptic phosphopeptide from pp42/MAP kinase. The electrospray ionization mass spectrum of the pp42/MAP kinase phosphopeptide (~10 pmol) recorded on the triple quadrupole instrument shows $(M + 2H)^{2+}$, $(M + 3H)^{3+}$ and $(M + 4H)^{4+}$ ions at *m/z* 1153.0, 769.0 and 577.0 (average mass), respectively. The *m/z* value (average mass) for the phosphopeptide $(M + H)^{+}$ ion calculated from these data is 2305.0. The inset shows the relevant mol. wt region of a mass spectrum recorded on the phosphorylated tryptic peptide (~3−5 pmol) under particle bombardment conditions on the quadrupole Fourier transform instrument. The unresolved isotope cluster for the observed $(M + H)^{+}$ ion is centered at 2304.7.

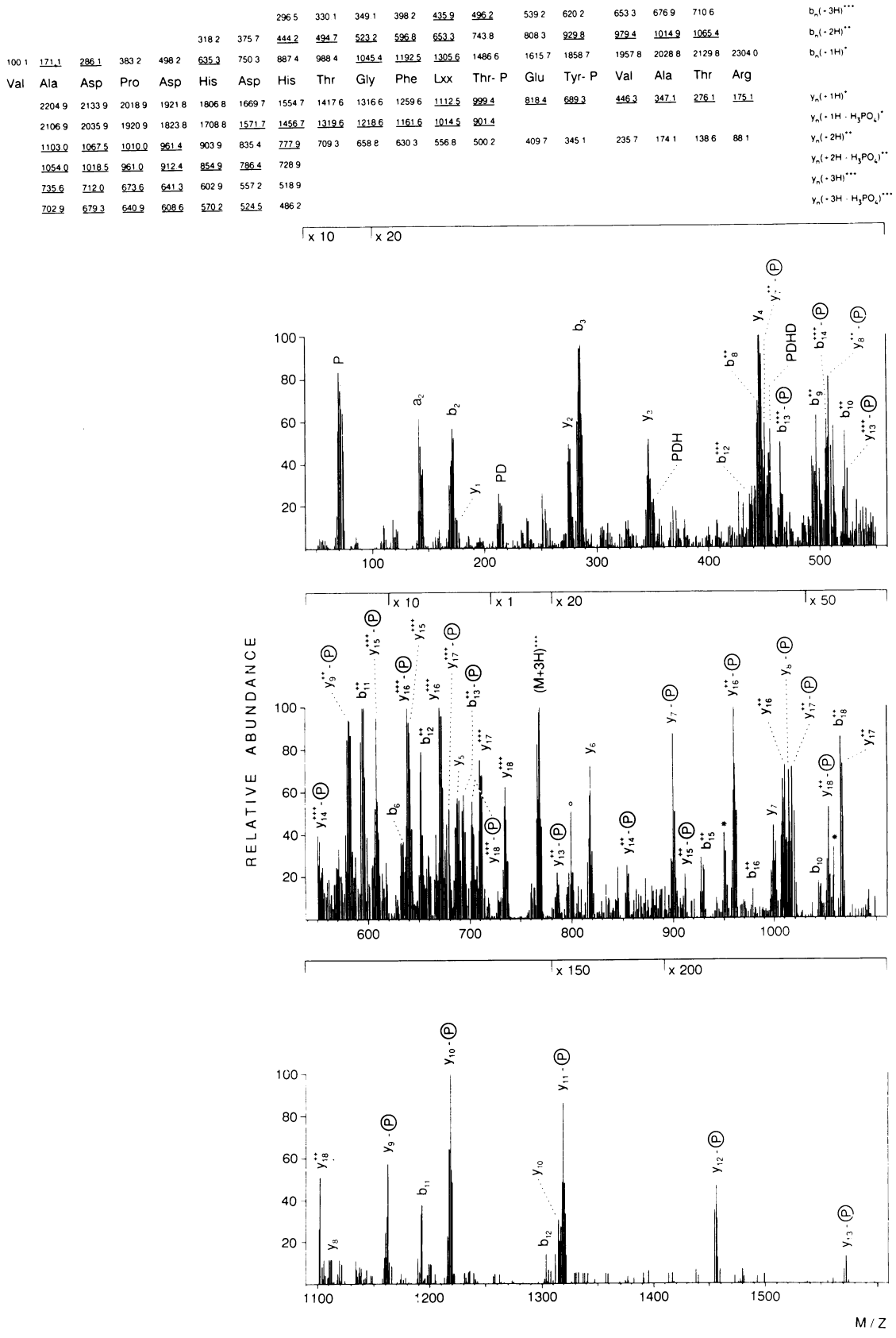

Fig. 5. Collision-activated dissociation (CAD) mass spectrum recorded on $(M + 3H)^{3+}$ ions (*m/z* 769.0, average mass) derived from the phosphorylated tryptic peptide of pp42/MAP kinase. Sample (~10 pmol) was ionized under electrospray conditions and analyzed on the TSQ-70 mass spectrometer. Predicted monoisotopic masses for fragments of types b, y and $y-H_3PO_4$ derived from the deduced sequence are shown above and below the sequence at the top of the figure; those observed in the spectrum are underlined. The abbreviations Thr-P and Tyr-P refer to phosphothreonine and phosphotyrosine, respectively; X refers to Ile or Leu. Fragment ions labeled with symbols -P, ° and * have lost the elements of phosphoric acid, water and ammonia, respectively. Fragments that result from internal cleavages at proline are labeled with the appropriate single letter codes (PPD, PDH and PDHD) to indicate the sequences of those fragments (Hunt *et al.*, 1986).

emphasized that the combination of HVE/TLC and reverse phase HPLC affords rapid purification of peptides by three very different modalities.

Peptide sequencing by mass spectrometry

Amino acid sequence analysis by the techniques of particle bombardment or electrospray ionization and collision-activated dissociation have been described in detail previously (Hunt *et al.*, 1986, 1991; Griffin *et al.*, 1989; Erickson *et al.*, 1990). The strategy employed here involved: (i) determination of the molecular mass of the peptide by both particle bombardment and the combination of microcapillary HPLC/electrospray ionization mass spectrometry; and (ii) fragmentation of the predominant $(M + nH)^{n+}$ ion in the electrospray spectrum by the technique of collision-activated dissociation. A collision-activated dissociation spectrum was also recorded on the ethyl ester derivative of the phosphopeptide to confirm assignments made to multi-charged fragment ions.

Mass spectra recorded on the MAP kinase phosphopeptide are displayed in Figures 4 and 5. Shown in the inset of Figure 4 is the relevant region of a mass spectrum recorded on the Fourier transform mass spectrometer. To obtain these data, one-tenth of the purified peptide (~3–5 pmol) was desorbed from a thioglycerol matrix under bombardment with 6 keV Cs^+ ions. The resulting low resolution spectrum shows an unresolved isotope cluster for the $(M + H)^+$ ion centered at *m/z* 2304.7. The electrospray ionization mass spectrum of the phosphopeptide (~10 pmol) (Figure 4) recorded on the triple quadrupole instrument shows $(M + 2H)^{2+}$, $(M + 3H)^{3+}$ and $(M + 4H)^{4+}$ ions at *m/z* 1153.0, and 769.0 and 577.0 (average mass) respectively. The *m/z* value (average mass) for the phosphopeptide $(M + H)^+$ ion calculated from these data is 2305.0. Observation of the $(M + 4H)^{4+}$ ion suggests that the peptide contains at least four basic sites (N-terminus plus Lys, Arg and/or His residues).

An electrospray ionization mass spectrum recorded on the ethyl ester of the MAP kinase phosphopeptide showed $(M + 2H)^{2+}$, $(M + 3H)^{3+}$ and $(M + 4H)^{4+}$ ions at *m/z* 1222.9, 815.6 and 612.0, respectively (data not shown). An *m/z* value of 2444.7 (average mass) for the $(M + H)^+$ ion is calculated from these data. Conversion of a carboxylic acid group to the corresponding ethyl ester increases the mass of the molecule by 28 daltons. Since the observed mass shift for the phosphopeptide $(M + H)^+$ ion is 140 daltons, five carboxylic acid groups (C-terminus plus four acidic residues) must be present in the molecule.

To determine the amino acid sequence and phosphorylated residues in the MAP kinase tryptic peptide, $(M + 3H)^{3+}$ ions (*m/z* 769.0) in the electrospray ionization spectrum were subjected to collision-activated dissociation (CAD) on the triple quadrupole mass spectrometer. Sample (~10 pmol) was injected into the electrospray ionization source directly from a microcapillary HPLC column as has been described previously (Hunt *et al.*, 1991). The resulting CAD spectrum and the sequence of amino acids deduced from it are shown in Figure 5. Predicted monoisotopic masses for fragment ions of type b and type y (Hunt *et al.*, 1986) are shown above and below the sequence in Figure 5; those observed in the spectrum are underlined.

Assignment of charge to a particular ion in the CAD spectrum of a multiply charged parent ion is greatly facilitated by recording a CAD spectrum on the same multiply charged ion from the corresponding peptide ethyl ester. Conversion of the peptide sample to its ethyl ester increases the mass of any particular fragment by 28, 14 and 9.3 daltons per carboxylic acid group for ions having a charge of +1, +2 and +3, respectively. Partial elimination of small neutral molecules such as ammonia, water and, in this case, phosphoric acid (98 daltons) also facilitates assignment of charge to a particular fragment. Loss of phosphoric acid generates daughter ions separated by 98, 49 and 32.7 daltons from parent ions having a charge of +1, +2 and +3, respectively. Both of the above approaches were employed to assign charges to the fragments in Figure 1.

Fragments of type y carrying up to three positive charges dominate the CAD spectrum (Figure 5). Ions of this type all contain the C-terminus plus 1, 2, 3, etc. additional residues (Hunt *et al.*, 1986). Subtraction of *m/z* values for any two fragments that differ by a single amino acid, NHCH(R)CO, and have the same number of charges generates a value that specifies the mass and thus the identity of the extra residue in the larger fragment.

Residues 12–19 in the MAP kinase phosphopeptide were deduced from fragments of type y that carry a single positive charge. Amino acids Leu and Ile are assigned as Lxx in the figure because they have the same mass and cannot be distinguished on the triple quadrupole instrument. Mass separations between y_7 and y_6 (181 daltons) and between y_5 and y_4 (243 daltons) are those corresponding to phosphothreonine and phosphotyrosine, respectively, and thus locate the two sites of phosphorylation as Thr13 and Tyr15 in the MAP kinase phosphopeptide. Note that y_7 and y_8 ions, both of which contain the phosphothreonine residue, undergo elimination of phosphoric acid (98 daltons) to form fragments at *m/z* 901 and 1014, respectively. This process is characteristic of phosphothreonyl or phosphoseryl residues, but has not been observed with phosphotyrosyl residues. Elimination of phosphoric acid from fragments of type y provides a series of abundant ions that defines the sequence of residues 7–13 in the MAP kinase phosphopeptide. Calculated *m/z* values for these fragments are shown on the second line below the sequence in Figure 5.

The remainder of the amino acid sequence in the phosphopeptide, residues 1–7, was deduced from ions of type y that carry either two or three positive charges. Note that the $y_{13}-H_3PO_4$ ion contains three basic groups (Arg19, His8 and an N-terminus) that can accept protons and form ions of type $y^{2+}-H_3PO_4$ and $y^{3+}-H_3PO_4$; these are observed at *m/z* 786.4 and 524.5, respectively. Residues 1–7 in the phosphopeptide sequence were assigned by locating additional members of this +2 and +3 series that are shifted to higher mass by half and one-third the normal amino acid residue masses, respectively. Fragments of this type that are observed in the spectrum appear on lines four and six below the sequence in Figure 5. We conclude that the MAP kinase tryptic phosphopeptide has the sequence VADPDHDHTGFXTEYVATR, where X is either Leu or Ile. The phosphorylated residues are Thr13 and Tyr15.

Discussion

Sequence of the phosphopeptide

MAP kinase requires phosphorylation both on tyrosine and on threonine for enzymatic activity (Anderson *et al.*, 1990).

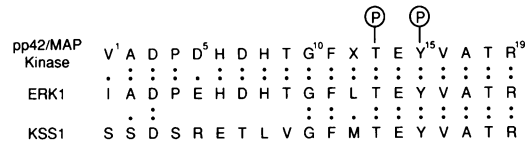

Fig. 6. Comparison of the MAP kinase tryptic phosphopeptide sequence with the sequences from homologous regions of ERK1- and KSS1-encoded kinases. The sequence of the pp42/MAP kinase peptide containing the regulatory phosphorylation sites is shown aligned with predicted sequences from ERK1 (residues 178–196) and KSS1 (residues 171–189). Two dots indicate identity with residues in pp42/MAP kinase; one dot indicates conservative differences. The residue at position 12 indicated as X (Leu or Ile) is Leu, based on DNA sequence analysis (Her *et al.*, unpublished data; see Discussion).

We have found that both of these phosphorylations occur on a single phosphopeptide, and have determined the sequence and sites of phosphorylation of this regulatory peptide. The structure of the phosphopeptide is compared in Figure 6 with partial amino acid sequences based on cDNAs for two serine/threonine kinases, ERK1 (Boulton *et al.*, 1990a) and KSS1 (Courchesne *et al.*, 1989). ERK1 is a partial rat cDNA for an insulin-stimulated microtubule-associated protein-2 kinase which shares some properties with pp42/MAP kinase, including rapid activation prior to S6 kinase *in vivo* and stimulation of an S6 kinase activity *in vitro* (Boulton *et al.*, 1990b). The protein encoded by ERK1 is closely related to KSS1, a yeast 42 kd serine/threonine kinase whose over-expression allows yeast variants permanently arrested by pheromones to escape arrest and re-enter the cell cycle. Escape from pheromone-induced arrest in yeast is in some respects biologically parallel to mitogen-stimulated transition from G_0 to G_1 in animal cells, an event which is associated with MAP kinase activation. Thus, these enzymes display conservation of both sequence and physiology.

The sequence of the tryptic phosphopeptide of murine pp42/MAP kinase is similar but not identical to a tryptic peptide within ERK1. Two conservative amino acid replacements (out of 19 residues) distinguish the sequences: Ile for Val1 and Glu for Asp5. In theory, these differences could be due to species-specific variations, since we have purified pp42/MAP kinase from mouse cells and the sequence deduced by Boulton *et al.* (1990a) is for a rat protein. However, we have isolated clones homologous to ERK1 from a mouse 3T3 cDNA library, and have determined their nucleotide sequences in the region corresponding to the phosphopeptide whose amino acid sequence we describe here. We obtained several clones with sequences identical to the rat ERK1, and one with the sequence shown in Figure 6 for MAP kinase. Therefore, we suggest that the ERK1 sequence encodes a different member of the MAP kinase family than the pp42/MAP kinase that we have characterized here and in previous reports (Ray and Sturgill, 1988a; Rossomando *et al.*, 1989; Erickson *et al.*, 1990).

Several protein kinases are activated following mitogen stimulation of cultured mammalian cells, including at least one enzyme, pp44, which is closely related to pp42/MAP kinase and to a 44 kd kinase (Rossomando,A.J., Sanghera,J.S., Marsden,L.A., Pelech,S.L., Weber,M.J. and Sturgill,T.W., manuscript in preparation) identified by Pelech and co-workers in maturing sea star oocytes (Sanghera *et al.*, 1990). These related protein kinases can be resolved from each other chromatographically and electrophoretically (Ahn and Krebs, 1990a,b; Rossomando *et al.*, manuscript in preparation); however, because characterization of the protein encoded by ERK1 has not yet appeared, we have no biochemical basis for determining whether this protein is likely to be pp44/MAP kinase or some other kinase. Moreover, because the 'complete nucleotide sequence' of the cDNA described by Boulton *et al.* (1990a) did not include the 5′ coding region of the gene, it is also not possible on genetic grounds to know the size of the protein encoded by ERK1. Determination of the structural and regulatory relationships among ERK1, pp42/MAP kinase and other members of the MAP kinase family will require the generation of molecular and immunological reagents specific for each member of the family.

The sequence TEYVATR containing the phosphorylation sites in pp42/MAP kinase is perfectly conserved in ERK1 and yeast KSS1. This raises the interesting possibility that these other enzymes are also regulated by phosphorylation at these sites, perhaps by kinases with similar specificity.

Regulation of MAP kinase by dual phosphorylations

Multi-phosphorylation is an established paradigm for regulation of enzyme function, permitting integration of signals from different signalling pathways (Roach, 1990). A frequent hallmark of such regulation is a grouping together of the regulatory phosphorylation sites. For some proteins, e.g. glycogen synthase, one purpose of phosphorylation at multiple sites may also be to produce a graded increase in response (Roach, 1990). pp42/MAP kinase and $p34^{cdc2}$ are regulated in a manner that suggests an additional function for regulation by multi-phosphorylation, namely that of a fail-safe control mechanism, as outlined below.

Although much remains unexplained regarding the phosphorylation state(s) of $p34^{cdc2}$, cyclins, and their complex, it is clear that mammalian $p34^{cdc2}$ is inactivated by phosphorylation of either tyrosine (Tyr15) or threonine (Thr14) within the ATP binding site (Morla *et al.*, 1989; Gould and Nurse, 1989; P.Nurse and C.Norbury, personal communication). Thus, phosphorylation occurs at adjacent tyrosine and and threonine sites, and both residues must be dephosphorylated to achieve activation of $p34^{cdc2}$. The use of two phosphorylations for inactivation, each of which alone suffices, fulfills the requirements for a fail-safe control mechanism.

Murine pp42/MAP kinase is also regulated by tyrosine and threonine phosphorylations, but in an opposite sense. Phosphorylation of both tyrosine and threonine residues is required for activation (Anderson *et al.*, 1990). We have demonstrated here that the regulatory phosphorylation sites in pp42/MAP kinase are separated by only one amino acid. This arrangement is entirely consistent with regulation of enzyme activity by dual phosphorylations, and in this respect parallels the close apposition of regulatory sites present in $p34^{cdc2}$.

The close proximity of these regulatory phosphorylations in MAP kinase could be a consequence of hierarchical phosphorylations, in which the initial phosphorylation creates a site for phosphorylation by a secondary kinase. Such 'site-permissive' phosphorylations necessarily result in closely spaced phosphorylations. For example, phosphorylations of Ser/Thr residues separated by three or two

residues result from secondary phosphorylations catalyzed by glycogen synthase kinase III and casein kinase I, respectively (Flotow and Roach, 1989). Site permissive effects, due to either primary threonine or tyrosine phosphorylation, must be considered among plausible mechanisms for MAP kinase activation. Alternatively, these phosphorylations could occur independently, possibly via different signalling pathways. Determination of the order, if any, of the tyrosine and threonine phosphorylations is needed to resolve this issue.

We have considered the possibility that the threonine phosphorylation of MAP kinase results from autophosphorylation. The threonine and tyrosine phosphorylation sites in MAP kinase are close to the conserved APE sequence (Her,J.-H., Wu,J., Sturgill,T.W. and Weber,M.J., unpublished) in kinase subdomain VIII, a region in which protein kinase autophosphorylations, some activating, are known to occur (Hanks *et al.*, 1988). However, MAP kinase is inactivated by phosphatase 2A (Anderson *et al.*, 1990) and does not regain activity upon reincubation with ATP/Mg and okadaic acid (N.Anderson and T.Sturgill, unpublished data), suggesting that autophosphorylation is not involved in this regulation. The sequence of the regulatory region for MAP kinase further mitigates against autophosphorylation as the source of the regulatory threonine phosphate. We have identified the specific site in bovine MBP phosphorylated by MAP kinase as Thr97, with partial sequence -Thr-Pro-Arg-Thr97-Pro-Pro-Pro- (Erickson *et al.*, 1990). The regulatory site phosphorylated in MAP kinase has no obvious resemblance to the MBP site, as would be expected were autophosphorylation the responsible mechanism.

As yet the kinases responsible for phosphorylation and activation of MAP kinase have not been identified. The phosphorylated threonine does not resemble recognition sites for any of the extensively characterized serine/threonine kinases (Kemp and Pearson, 1990). Consensus recognition sequences for tyrosine kinases have not been as well defined (Kemp and Pearson, 1990), although one common feature is the presence of acidic residue(s) near the phosphorylated tyrosine, as found here. The phosphorylated tyrosine is not found within a motif which resembles the insulin receptor autophosphorylation site (Boulton *et al.*, 1990a). While direct activation of MAP kinase by a receptor tyrosine kinase is still an attractive hypothesis, there is as yet no evidence to support it. Attempts to demonstrate phosphorylation of MAP kinase by the insulin receptor are so far negative (B.Ray, N.Anderson and T.Sturgill, unpublished data). Finally, it is conceivable that a single kinase with dual specificity for both threonyl and tyrosyl residues is responsible for phosphorylating MAP kinase (Stern *et al.*, 1991). Knowledge of the regulatory phosphorylation sites in MAP kinase will augment approaches available for identifying the regulatory kinases and for dissecting the upstream pathways responsible for their activation.

Materials and methods

Materials

Phorbol 12,13 dibutyrate (PDB) and bovine MBP were purchased from Sigma. The sources of other reagents have been given (Ray and Sturgill, 1988a; Anderson *et al.*, 1990; Erickson *et al.*, 1990). Stock cultures of EL4.IL-2 thymoma cells were obtained from Dr Julianne Sando, University of Virginia. All chromatography media were purchased from Pharmacia-LKB.

MAP kinase assay

MAP kinase activity was measured using bovine MBP as substrate, as described previously (Erickson *et al.*, 1990). During the development of procedures for isolating MAP kinase, the phosphorylated protein (pp42) was detected by Western blotting with anti-phosphotyrosine antibodies (Rossomando *et al.*, 1989).

Cell culture, mitogen stimulation and preparation of cell extract

EL4 cells were grown to a density of $1-2 \times 10^6$ cells/ml in spinner culture (Jensen and Sando, 1987). Cells from spinner cultures (5–6 l) were collected by centrifugation, washed twice with 800 ml Kreb's Ringer bicarbonate (KRB)–HEPES buffer (37°C), then resuspended to 2×10^7 cells/ml in KRB–HEPES and incubated for 1–2 h (Ray and Sturgill, 1988a) with gentle rocking. Cells were stimulated at 37°C for 10 min with 650 nM phorbol dibutyrate (PDB) (final concentration) and were then quickly chilled by addition of 0.5–1 vol finely crushed, frozen KRB–HEPES with vigorous swirling; all subsequent manipulations were performed at 0–2°C. Cells were pelleted and resuspended (2×10^7 cells/ml) in homogenization buffer (Ray and Sturgill, 1988a), containing 1 mM Na_3VO_4, 0.4 mM PMSF, 1 μM pepstatin and 1 μg/ml leupeptin and were disrupted by nitrogen cavitation (Jensen and Sando, 1987). The supernatant after centrifugation (25 000 *g*, 15 min), was adjusted to 10% ethylene glycol (v/v) and stored at −70°C as the starting material ('S25 extract').

MAP kinase purification

Preparative-scale isolation of pp42/MAP kinase from the S25 extract was accomplished by a combination of anion exchange and hydrophobic interaction chromatography, representing an adaptation of the previously described analytical procedure (Ray and Sturgill, 1988a). To generate sufficient material for sequencing, MAP kinase was purified from a total of 1.3×10^{10} cells (12.5 l of cultured cells) in five separate batches, and the enzyme resulting from these preparations was pooled; the volumes given below are representative of those used for each preparation. MAP kinase was enriched from the S25 lysate (150 ml) by batch adsorption to phenyl-Sepharose (0.4 g moist cake/ml S25) which had been equilibrated in buffer C (Ray and Sturgill, 1987) containing 10% ethylene glycol. The matrix was washed twice with 100 ml buffer C containing 10% ethylene glycol, followed by two 100 ml washes with buffer C containing 35% ethylene glycol. MAP kinase was then eluted with 150 ml buffer C containing 60% ethylene glycol. The eluate was diluted to 30% ethylene glycol with buffer C, applied to a preparative Mono Q column (HR10/10), washed with 120 ml of buffer C and eluted with a 240 ml, 0–1 M NaCl gradient in buffer C (2 ml/min). The peak fractions of MBP phosphotransferase activity, eluting at ~0.2 M NaCl, were pooled and stored at −70°C after addition of ethylene glycol (10% v/v, final concentration). The Mono Q pool (20 ml) was fractionated by preparative phenyl-Superose (HR10/10 column) chromatography (see Figure 2), using conditions scaled up 8-fold relative to those described for analytical phenyl-Superose chromatography (Ray and Sturgill, 1988a). Fractions (4 ml) were collected into tubes containing 0.1 vol of 0.2% Tween-20, and the fractions containing pp42/MAP kinase were determined by the MBP kinase assay (Erickson *et al.*, 1990) and in some cases by immunoblotting with anti-phosphotyrosine antibodies as well (Rossomando *et al.*, 1989).

Preparation of [^{32}P]MAP kinase

[^{32}P]MAP kinase was prepared from 3×10^8 EL4.IL-2 cells labeled for 2 h with 50 mCi $^{32}P_i$ in 20 ml, following the procedure previously described (Ray and Sturgill, 1988a). [^{32}P]MAP kinase was the predominant phosphoprotein in the pooled phenyl-Superose peak as detected by autoradiography following SDS–PAGE (see Results, Figure 1A), typically representing 12–25% of the radioactivity in the phenyl-Superose pool (estimated by excising and counting the ^{32}P-labeled 42 kd band, in comparison to the total c.p.m. applied). The major radioactivity, besides the MAP kinase peak, migrated at the dye front, and presumably consisted of lipids, P_i and other low M_r material which was not fully separated from MAP kinase during the initial isolation. A typical preparation yielded 30 000 c.p.m. (Cerenkov) [^{32}P] of MAP kinase.

Preparative SDS gel electrophoresis

Pooled MAP kinase from five preparative purifications (88 ml total volume) was combined with purified [^{32}P]MAP kinase from 3×10^8 cells. The sample (90 ml) was divided into six portions and concentrated in Amicon centripreps with a 10 000 mol. wt cutoff, followed by precipitation with trichloroacetic acid/deoxycholate as described (Rossomando *et al.*, 1989). The precipitate was resuspended with 500 μl SDS sample buffer and electrophoresed on a preparative 10% polyacrylamide gel; the upper (cathode) buffer contained 0.1 mM thioglycolate (Hunkapiller *et al.*, 1983). The

position of pp42 was determined by autoradiography of the wet gel, and the pp42 band was excised from the gel, the slice was crushed and the protein was extracted with 25 mM *N*-ethylmorpholine, adjusted to pH 7.7 with trifluoroacetic acid. Sample was dried in a Savant Speedvac, resuspended in 200 μl of 200 mM ammonium bicarbonate, and precipitated with trichloroacetic acid following addition of 50 μg of poly(Glu/Tyr) (Sigma) as carrier.

Peptide isolation

Proteolytic digestions for peptide mapping or sequencing were performed as described previously (Erickson, 1990), with the following modifications. Samples for sequencing were suspended in 200 μl of 50 mM ammonium bicarbonate, pH 8.0, and digested at 37°C with sequencing grade trypsin (Boehringer-Mannheim). Trypsin (10 μg) was added for 4 h, an additional 10 μg was added for another 4 h, and then a final 10 μg was added and the sample incubated overnight.

The peptide mapping was as described previously (Erickson *et al.*, 1990), except for the following preparative modifications. For sequencing, the products of tryptic digestion were dissolved in pH 1.9 buffer, and half of the sample was applied to each of two thin-layer plates. Prior to electrophoresis, the samples were 'pre-run' in the chromatography dimension (6 cm) to minimize streaking (Whittaker and Moss, 1981). The phosphopeptide was eluted from the cellulose with TLC solvent (butanol:pyridine: acetic acid:water, 75:50:15:60) and dried.

Prior to sequencing, the phosphopeptide was dissolved in 5 μl formic acid, diluted to 100 μl with water and further purified by HPLC as described previously (Erickson, 1990), but using a BU-300 microbore column (2.1 × 30 mm, Applied Biosystems). Absorbance of the column effluent was monitored at 214 nm and ^{32}P in each fraction was determined by Cerenkov counting.

Mass spectrometry

Mass spectra were recorded on both a TSQ-70 triple quadrupole instrument (Finnigan-MAT, San Jose, CA) equipped for electrospray ionization and on a quadrupole Fourier transform instrument constructed at the University of Virginia (Hunt *et al.*, 1987). Operation of the latter instrument has been described previously (Hunt *et al.*, 1987). Samples for analysis on the TSQ-70 instrument were dissolved in 1 μl formic acid, diluted to 10 μl with water and aliquots were injected into the electrospray ionization source from a fused-silica, microcapillary-HPLC column with an inside diameter of 75 μm and a length of 70 cm (Hunt *et al.*, 1991). The last 10 cm of the column was filled with C-18 packing material. Phosphopeptide was eluted with a 10 min gradient of 0–80% acetic acid (0.5%)/acetonitrile flowing at a rate of 1–2 μl/min. Peptide ethyl esters were prepared as described (Hunt *et al.*, 1986).

Phosphoamino acid analysis

Phosphoamino acid analysis was performed by one-dimensional electrophoresis (60 min, 1 kV) at pH 3.5 following a 1 h partial hydrolysis as described previously (Cooper *et al.*, 1983).

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References

Ahn,N.G. and Krebs,E.G. (1990a) *J. Biol. Chem.*, **265**, 11487–11494.
Ahn,N.G. and Krebs,E.G. (1990b) *J. Biol. Chem.*, **265**, 11495–11501.
Anderson,N.G., Maller,J.L., Tonks,N.K. and Sturgill,T.W. (1990) *Nature*, **343**, 651–653.
Boulton,T.G., Yancopoulos,G.D., Gregory,J.S., Slaughter,C., Moomaw,C., Hsu,J. and Cobb,M.H. (1990a) *Science*, **249**, 64–67.
Boulton,T.G., Gregory,J.S., Jong,S.-M., Wang,L.-H., Ellis,L. and Cobb,M.E. (1990b) *J. Biol. Chem.*, **265**, 2713–2719.
Cooper,J.A., Sefton,B.M. and Hunter,T. (1983) *Methods Enzymol.*, **99**, 387–405.
Courchesne,W.E., Kunisawa,R. and Thorner,J. (1989) *Cell*, **58**, 1107–1119.
Ely,C.M., Oddie,K., Litz,J.S., Rossomando,A.J., Kanner,S.B., Sturgill,T.W. and Parsons,S.J. (1990) *J. Cell Biol.*, **110**, 731–742.
Erickson,A.K., Payne,D.M., Martino,P.A., Rossomando,A.J., Shabanowitz,J., Weber,M.J., Hunt,D.F. and Sturgill,T.W. (1990) *J. Biol. Chem.*, **265**, 19728–19735.
Erikson,R.L., Alcorta,D., Bedard,P.A., Blenis,J., Biemann,H.-P., Erikson,E., Jones,S.W., Maller,J.L., Martins,T.J. and Simmons,D.L. (1988) *Cold Spring Harbor Symp. Quant. Biol.*, **53**, 143–151.
Flotow,H. and Roach,P.J. (1989) *J. Biol. Chem.*, **264**, 9126–9128.
Gould,K.L. and Nurse,P. (1989) *Nature*, **342**, 39–45.
Gregory,J.S., Boulton,T.G., Sang,B.-C. and Cobb,M.H. (1989) *J. Biol. Chem.*, **264**, 18397–18401.
Griffin,P.R., Kumar,S., Shabanowitz,J., Charbonneau,H., Namkung,P.C., Walsh,K.A., Hunt,D.F. and Petra,P. (1989) *J. Biol. Chem.*, **264**, 19066–19075.
Hanks,S.K., Quinn,A.M. and Hunter,T. (1988) *Science*, **241**, 42–52.
Hunkapiller,M.W., Lujan,E., Ostrander,F. and Hood,L.E. (1983) *Methods Enzymol.*, **91**, 227–236.
Hunt,D.F., Yates,J.R., III, Shabanowitz,J., Winston,S. and Hauer,C.R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 6233–6237.
Hunt,D.F., Shabanowitz,J., Yates,J.R., III, Zhu,N.Z., Russell,D.H. and Castro,M.E. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 620–623.
Hunt,D.F., Alexander,J.E., McCormack,A.L., Martino,P.A., Michel,H., Shabanowitz,J., Sherman,N., Moseley,M.A., Jorgenson,J.W. and Tomer,K.B. (1991) In Villafranca,J.J., (ed.), *Current Research in Protein Chemistry Techniques, Structure and Function*. Academic Press, New York, in press.
Jensen,D.E. and Sando,J.J. (1987) *Cancer Res.*, **47**, 3868–3872.
Kemp,B.E. and Pearson,R.B. (1990) *Trends Biochem. Sci.*, **15**, 342–346.
L'Allemain,G., Sturgill,T.W. and Weber,M.J. (1991) *Mol. Cell. Biol.*, **11**, 1002–1008.
Morla,A.O., Draetta,G., Beach,D. and Wang,J.Y. (1989) *Cell*, **58**, 193–203.
Ray,L.B. and Sturgill,T.W. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1502–1506.
Ray,L.B. and Sturgill,T.W. (1988a) *J. Biol. Chem.*, **263**, 12721–12727.
Ray,L.B. and Sturgill,T.W. (1988b) *Proc. Natl. Acad. Sci. USA*, **85**, 3753–3757.
Roach,P.J. (1990) *FASEB J.*, **4**, 2961–2968.
Rossomando,A.J., Payne,D.M., Weber,M.J. and Sturgill,T.W. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6940–6943.
Sanghera,J.S., Paddon,H.B., Bader,S.A. and Pelech,S.L. (1990) *J. Biol. Chem.*, **265**, 52–57.
Stern,D.F., Zheng,P., Beidler,D.R. and Zerillo,C. (1991) *Mol. Cell. Biol.*, **11**, 987–1001.
Sturgill,T.W., Ray,L.B., Erikson,E. and Maller,J.L. (1988) *Nature*, **334**, 715–718.
Whittaker,R.G. and Moss,B.A. (1981) *Anal. Biochem.*, **110**, 56–60.

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Note added in proof

An activating phosporylation in $p34^{cdc2}$ (Thr167) is similarly located upstream of the APE kinase motif (see Lewin,B. *Cell*, **61**, 743–752. Characterization of the ERK1 protein kinase has now been published (Boulton,T.G., Gregory,J.S. and Cobb,M.H. (1991) *Biochemistry*, **30**, 278–286); most of the data therein are consistent with the ERK1 protein being pp44/MAP kinase.